[CANCER RESEARCH (SUPPL.) 50, 5671s–5674s, September 1, 1990]

Oncogenes in Hematopoietic and Hepatic Fish Neoplasms[1]

Rebecca J. Van Beneden,[2] Kelly W. Henderson, Donald G. Blair, Takis S. Papas, and Henry S. Gardner

Duke University Marine Laboratory, Beaufort, North Carolina 28516, and Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710 [R. J. V. B.]; Department of the Army, US Army Biomedical Research and Development Laboratory, Ft. Detrick, Frederick, Maryland 21701 [H. S. G.]; Program Resources, Inc., Frederick, Maryland 21701 [K. W. H.]; and Laboratory of Molecular Oncology, Frederick Cancer Research Facility, Frederick, Maryland 21701 [D. G. B., T. S. P.]

Abstract

Neoplastic transformation of cells has often been associated with changes in cellular oncogenes. While much information has been collected in mammalian systems, relatively little is known about the molecular basis of tumor progression in lower vertebrates. For our studies, tumors were collected from feral northern pike (*Esox lucius*) from Ostego Lake, MI, where the local population exhibited a 15% incidence of large external lymphomas. In laboratory studies, tumors were induced under controlled conditions by known mammalian carcinogens in the Japanese medaka (*Oryzias latipes*), a small aquarium fish widely used in carcinogenicity studies. DNA isolated from these tumors was assayed for transforming sequences by transfection into NIH3T3 cells. DNAs from the northern pike lymphomas and the chemically induced tumors in the medaka were able to transform NIH3T3 cells and induce tumors in athymic mice. The results of our studies to date are summarized here, together with the current status of oncogene activation in other fish systems.

Introduction

Teleost fish constitute the largest and most diverse class of vertebrates, with over 20,000 known species. Their diversity and place in the phylogenetic tree make them ideal subjects for comparative carcinogenesis studies which may allow more insight into basic mechanisms than studies limited to mammalian models alone. Only relatively recently have fish begun to gain importance for use in carcinogenicity studies. Fish tumor investigation is now seen as an integral part of the basic, biological approach to elucidating common mechanisms of cancer at different phylogenetic levels. Neoplasms have been reported in virtually all the major organs and cell types in many species of fish. While the structure of some organs in fish differs considerably from those in mammals, tumors are often very similar histologically and may be classified by criteria similar to those used in human tumor diagnosis.

The high incidence of tumors in feral fish and chemical induction of tumors in fish in the laboratory have been well documented (1, 2). However, very little is known about the molecular basis of carcinogenesis in these animals. It is important to emphasize that, even though exposure to chemical carcinogens is highly correlated with tumor incidence, it is extremely difficult to prove causality. One way that the etiology of these tumors may be assessed is through the application of molecular methods. Central to such an evaluation is the identification of oncogene sequences and characterization of their role in tumor formation.

Ironically, probably the first "oncogene" ever to be described was in a fish. Following the first report of melanoma formation in swordtail-platyfish (*Xiphophorus*) hybrids in 1928, a series of elegant, classical genetic studies by Gordon, Anders, Schartl, and others led to the postulation of a "tumor" gene, *tu* (3). *tu* was recently cloned and has been identified as an oncogene. It codes for a novel RTK,[3] closely related to the receptor for epidermal growth factor (4). The first oncogenes from fish identified by cloning and sequence analysis were the *myc* gene in rainbow trout (5) and the *ras* gene from goldfish (6). The isolation and expression of several additional oncogenes have since been reported from *Xiphophorus* (7). The elevated expression of the *src* gene was noted in *Xiphophorus* melanomas, although it was demonstrated not to be directly involved in tumor formation (8).

More recently, McMahon *et al.* (9) reported a point mutation in a c-K-*ras* gene from a liver tumor in winter flounder (*Pseudopleuronectes americanus*), identified through NIH3T3 transfection analysis. They observed a single G → T base transversion in the second base of the 12th codon, the same type of mutation reported in *ras* genes from some chemically induced rodent tumors (10). Wirgin *et al.* (11) have also reported an activated K-*ras* in Atlantic tomcod (*Microgadus tomcod*) liver tumors.

Work in our laboratory indicated that DNAs isolated from tumors in feral northern pike (*Esox lucius*), as well as DNAs from chemically induced tumors in laboratory medaka (*Oryzias latipes*), are also able to transform NIH3T3 cells (12, 13).

In this paper, we report the results of our transfection studies and summarize the studies to date on fish oncogenes in hematopoietic and hepatic neoplasms.

Materials and Methods

Fish. Tumors were collected from northern pike from Ostego Lake, Gaylord, MI. Over 20% of the fish in this area exhibited large external lesions, identified as lymphomas.

Tumors were chemically induced in the medaka in two different experiments. In the first, a group of 14-day-old medaka fry were exposed for 48 h to 200 mg/liter of DEN dissolved in water. Animals were transferred to aquaria containing clean water for a period of 360 days. They were then removed, anesthetized, and sacrificed. Livers from these animals were excised, and small portions of each liver were individually fixed in Bouin's solution and subsequently stained with hematoxylin-eosin for histopathological assessment. The remaining tissue was frozen immediately in liquid nitrogen and stored at −70°C until DNA was extracted.

In a second experiment, 8-day-old medaka embryos were exposed prior to hatch for 4 h to 50 mg/liter of MAMac dissolved in water. A single animal was sacrificed, and a grossly observable tumor was removed from the caudal peduncle. This tissue was then treated as described above for the DEN-induced lesions.

Transfection Analysis. DNA was prepared by quick Dounce homogenization (12). Integrity of the DNA was determined by analysis on a 0.35% agarose gel, where it migrated more slowly than did intact λ-phage DNA. The transfection procedure was modified from that of Graham and van der Eb (14) and is presented elsewhere (12). Briefly, high-molecular-weight fish DNA was cotransfected with pSV$_2$neo in the presence of calcium phosphate into NIH3T3 cells (strain 490N3T). Cells were grown under drug selection with G418 (Geneticin; Gibco), pooled, and replated for three assays. Cells were grown to confluency and examined for foci of altered morphology. A second aliquot of the

[1] Presented at the "XIVth Symposium of the International Association for Comparative Research on Leukemia and Related Diseases," October 8–12, 1989, Vail, CO.

This work was supported in part by a Special Fellowship from the Leukemia Society of America, Inc., and NIH Grant ES03729 to R. J. V. B.

[2] To whom requests for reprints should be addressed, at the Duke University Marine Laboratory.

[3] The abbreviations used are: RTK, tyrosine kinase receptor; DEN, diethylnitrosamine; MAMac, methylazoxymethanol acetate.

same pool was examined for tumorigenicity by injection into athymic mice (15). A third aliquot was replated and grown in defined serum-free medium (QBSF; Quality Biologicals) in a colony selection assay. Foci and colonies were picked and expanded. Tumors were excised from nude mice. DNA was prepared as described earlier (12).

Characterization of Transformed Cells. Transformed cells were grown in soft agar to test for anchorage dependence (16). The maximum stringency necessary to detect oncogene sequences by Southern analysis in fish DNA from normal tissue was determined in pilot experiments. Transfected DNA was then analyzed on Southern blots (17) which were hybridized to known oncogene probes at the predetermined appropriate stringency. These were examined for homology of the fish transforming sequences. The presence of fish sequences in DNA from transformed cells was examined on Southern blots of transfectant DNA digests by hybridization of these to radiolabeled (18) high-molecular-weight medaka genomic DNA.

Results

Northern Pike Transfections. In primary transfection experiments, foci of transformed cells were detected in three of four plates that were transfected with DNA from pike tumors and grown in the presence of dexamethasone (Table 1). No foci were observed in control plates. Cells from individual foci were picked, expanded, and replated in a soft agar assay. They were able to form small colonies, characteristic of transformed mouse fibroblasts. Transformation efficiency increased in a secondary transfection assay. Cells from foci isolated from this transfection were also able to form colonies in soft agar, at a relatively faster rate than the primary transfectants (Table 2). No homology was observed to known oncogene probes on Southern blots of digested DNA from transfected cells (data not shown).

Medaka Transfections. Results of the primary and secondary transfection assays using medaka DNA are presented in Tables 3 and 4. DNA isolated from a DEN-induced cholangiocarcinoma was the most active in our assay, followed by DNA from animals with DEN-induced focal biliary hyperplasia. DNAs isolated from histologically normal or mildly abnormal, nonneoplastic liver from DEN-exposed fish were unable to transform NIH3T3 cells.

DNA from a MAMAc-induced rhabdomyosarcoma was unable to induce focus formation in the standard focus assay. However, three of four secondary transformants were able to grow in minimal media at very low serum levels, and two of these transformants were able to induce tumors in nude mice at frequencies and rates significantly higher than control cells (Table 4). Since only a single individual was examined, this experiment will be repeated.

In Southern analyses of DNA from foci cells, distinct bands which hybridized to radiolabeled medaka DNA were present in restriction digests of DNA from NIH3T3 cells transfected with fish tumor DNA (12). These bands were not present in DNA digests isolated from NIH3T3 cells alone or in NIH3T3 cells transfected with normal control fish DNA. In addition, in the original transfection of medaka cholangiocarcinoma DNA, two plates which were independently transfected with this DNA were both highly transformed. DNA isolated from foci from these two plates showed overlapping fish-specific fragments on Southern blots. Efficiency of transformation has increased in secondary (Table 4) and tertiary transfections (data not shown).

Southern blots prepared using DNA isolated from transfected cells and nude mouse tumors were hybridized to known oncogene probes at the appropriate stringency. There appears to be no homology to H-*ras*, K-*ras*, *neu*, *yes*, v-*erb*-B, *myc*, or *src* in DNA from any of the medaka transfected cells examined. DNAs from northern pike are currently being analyzed.

Table 1 *Primary transfection of northern pike DNA into NIH3T3 cells*

DNAs from normal northern pike liver tissue and malignant lymphomas were transfected into NIH3T3 cells via calcium phosphate precipitation (14). After 2 wk of drug selection with G418, cells were replated, grown to confluency, and scored for foci of altered morphology. Individual foci were picked, and cells were grown in soft agar and examined for anchorage dependence (16).

DNA source	Focus assay (foci/μg of DNA)		Relative colony growth in soft agar[a]
	DCF5[b]	DCF5 + DEX	
Lymphoma	0	0.19	+
Normal liver	0.01	0	–

[a] –, no growth; +, small colonies; ++, many colonies, comparable to positive control cells (*mos*- and H-*ras*-transformed NIH3T3 cells).
[b] DCF5, Dulbecco's modified Eagle's medium with 5% fetal calf serum; DCF5 + DEX, DCF5 supplemented with dexamethasone.

Table 2 *Colony formation in soft agar by cells transfected with northern pike DNA*

DNA isolated from primary transfectants was used in a secondary transfection assay of NIH3T3 cells (14). Foci were isolated and, along with control cells, were grown in soft agar to determine anchorage dependence (16).

Cells[a]	Relative colony growth[b]
TR13-16Aa (normal liver)	–
TR13-19Aa (lymphoma)	++
TR13-21Aa (lymphoma)	++
NIH3T3 cells	–
mos-transformed NIH3T3 cells	++
H-*ras*-transformed NIH3T3 cells	++

[a] Cells were expanded from foci isolated from a secondary transfection of northern pike tumor and control DNA as indicated.
[b] –, no growth; ++, many large colonies, comparable to positive control cells (*mos*- and H-*ras*-transformed cells).

Table 3 *Transformation efficiency of G418-selected cells in a primary transfection assay of medaka DNA*

DNAs isolated from normal medaka livers and livers from chemically exposed medaka were used to transfect NIH3T3 by calcium phosphate precipitation (14). Following G418 selection, cells were replated in a standard focus assay and examined for foci of altered morphology. A second aliquot of drug-selected cells was injected into nude mice (15). Cells from isolated foci were examined for anchorage dependence (16).

DNA source	Focus assay (foci/μg of DNA)		Nude mouse assay[a]	Growth in soft agar of isolated focus cells[b]
	DCF5[c]	DCF5 + DEX		
Untreated medaka controls	0.04	0.02	4/16	–
MAMAc-induced rhabdomyosarcoma	0.12	0.17	1/10	+
DEN-exposed fish				
Focal biliary hyperplasia	0.47	5.4	4/8	+
Histologically normal	0	0.04	4/12	–
Cholangiocarcinoma	0.26	4.9	8/8	+++
Histologically abnormal, nonneoplastic[d]	0.39	0.36	8/55	–

[a] Tumors at <5 wk postinjection.
[b] Growth relative to control cells (see Table 2).
[c] DCF5, Dulbecco's modified Eagle's medium with 5% fetal calf serum; DCF5 + DEX, DCF5 supplemented with dexamethasone. Data are presented as the average of 2 to 11 samples, as summarized from Van Beneden *et al.* (12).
[d] Includes spongiosis hepatis, bile duct hyperplasia, and bile duct ectasia.

Discussion

While the detailed mechanisms of tumorigenesis are unknown, increasing evidence suggests that genetic alterations of cellular oncogenes are in part responsible for the neoplastic transformation of cells. Methods of activation include point mutations, inappropriate gene expression, chromosomal translocation, and gene amplification. Although much work has been

[4] S. M. Robertson, unpublished data.

Table 4 *Summary of medaka secondary transfection analysis*

DNAs used for the secondary transfection were isolated from cells of the primary transfection as described in Table 3.

DNA source	Focus assay		Colony selection		
	DCF5[b]	DCF5 + DEX	QBSF medium	QBSF + 0.1% serum	Nude mouse assay[a]
Untreated medaka controls	0.57	0	–	–	2/16
MAMAc-induced rhabdomyosarcoma	0	0	–	+	8/16
DEN-induced focal biliary hyperplasia	0.77	0.53	–	–	10/16
DEN-induced cholangiocarcinoma	5.0	5.0	+	+	47/47

[a] Tumors at <5 wk postinjection. Data are summarized from Van Beneden *et al.* (12).
[b] DCF5, Dulbecco's modified Eagle's medium; DCF5 + DEX, DCF5 supplemented with dexamethasone; QBSF, Quality Biologicals Serum-free medium.

done with rodent, avian, and *Drosophila* models, very little attention has focused on teleosts. It wasn't until 1986 that the existence of cellular oncogenes in fish was confirmed by cloning and sequencing (Table 5). At that time, Nemoto *et al.* (6) presented the sequence of goldfish *ras*, and Van Beneden *et al.* (5) reported the sequence of rainbow trout c-*myc* (5). There was no evidence, however, for the involvement of either of these genes in any fish tumors (20, 21).

Results of our transfection assays indicate that DNA from fish tumors is able to transform NIH3T3 cells and induce tumors in nude mice. The DNA isolated from the DEN-induced cholangiocarcinoma was the most active in our transfection assays. Secondary transfectants induced tumors in nude mice in 1.5 to 2.5 wk. DNA from the MAMAc-induced rhabdomyosarcoma was unable to induce foci. However, cells transfected with this DNA were able to grow in low (1%) serum media and induced tumors in nude mice at a level significantly higher than control cells.

We have not yet identified these oncogenes. Previous studies (12) have shown that fish sequences were present in DNA from transformed cells. They do not, based on Southern analysis, appear to be homologous to the known oncogenes that we examined. We are currently cloning this gene from a transformed cell-derived library.

Table 5 *Cellular oncogenes reported in fish*

Species	DNA source	Oncogene	Reference
Xiphophorus sp. hybrids	Melanoma	*tu*	Ref. 4
	Brain, other normal organs, melanoma, squamous cell carcinoma, epithelioma, fibrosarcoma, retinoblastoma, rhabdomyosarcoma, mesenchymal tumor	c-*src*	Ref. 19
	Normal tissue	c-*yes* c-*erb*-B	Footnote 4
Rainbow trout (*Salmo gairdneri*)	Normal liver	c-*myc*	Ref. 5
Goldfish (*Carassius auratus*)	Normal liver Erythrophoroma-derived cell line	*ras*	Refs. 6 and 20
Winter flounder (*Pseudopleuronectes americanus*)	Liver tumors	K-*ras*	Ref. 9
Atlantic tomcod (*Microgadus tomcod*)	Liver tumors	K-*ras*	Ref. 11
Northern pike (*Esox lucius*)	Lymphoma	Unknown	Ref. 5 and this paper
Medaka (*Oryzias latipes*)	Cholangiocarcinoma	Unknown	Ref. 12

Other studies have used transfection analysis to address the role of oncogenes in fish tumors. McMahon *et al.* (9) reported an activated K-*ras* in liver tumors of winter flounder. In five fish examined, they observed a single base change in the 12th codon, a region of frequent mutation in activated mammalian *ras* genes (10). The authors speculated that the presence of an activated oncogene may indicate exposure of the fish to polycyclic aromatic hydrocarbons known to be abundant in sediments at the collection site. An activated K-*ras* gene has also been reported from transfection analysis of Atlantic tomcod tumors (11). The tomcod is also a bottom-dwelling fish. Adult fish of >1 yr old exhibit about a 90% incidence of neoplasia in the Hudson River (22). Southern blots of DNA from transfected cells showed a unique band that hybridized to K-*ras* which was not present in NIH3T3 or tomcod DNA digests. This gene has not been sequenced, and no evidence was presented to confirm its identity as a tomcod c-*ras*.

Melanoma production in *Xiphophorus* hybrids is perhaps one of the most interesting fish systems in which oncogenes have been detected. The evidence for the presence of an oncogene was accumulated through years of classical genetic analysis and, more recently, by molecular techniques. In hybrid fish (*X. maculatus* × *X. helleri*), the macromelanophores develop into malignant melanomas. This process results from abnormal regulation of the platyfish *tu* gene. *tu* has been known for some time to be a dominant tumor gene (macromelanophore locus) and is under the control of the regulatory gene *R* which acts as a tumor suppressor gene. Repeated backcrossing eliminates the regulatory control and deregulates *tu* expression. The *tu* gene has been recently cloned and sequenced (4). It has a high degree of sequence homology to the RTKs and especially to the receptor for human epidermal growth factor. In addition to the oncogenic *tu*, now renamed *Xmrk*, on the sex chromosomes, another copy was detected at the *INV* locus and is thought to be a protooncogene. Expression of the *Xmrk* is correlated with the degree of malignancy.

While the study of tumorigenesis in fish at the molecular level still lags behind that of higher vertebrates, the studies summarized here (Table 5) indicate that considerable progress has been made in recent years. Studies of oncogenes and tumor progression in teleosts promise to shed some light on basic mechanisms of carcinogenesis and may reveal novel oncogenes that may not be detected by the exclusive use of mammalian models.

Acknowledgments

The authors wish to express appreciation to Dr. Marilyn Wolfe of Experimental Pathology Laboratories, Herndon, VA, for her assistance in the histopathological assessment of the medaka samples. We appre-

ciate the aid of Dr. Eric May, University of Maryland, in the histopathological assessment of the northern pike samples. We are also grateful for the expert technical assistance in the conduct of the medaka bioassays provided by David Harvey and Robert Bishoff of the US Army Biomedical Research and Development Laboratory, Frederick, MD. We appreciate the invaluable help of the Department of Natural Resources, Gaylord, MI, in obtaining the northern pike.

References

1. Hoover, K. L. Use of small fish species in carcinogenicity testing. Natl. Cancer Inst. Monogr., *65:* 5–128, 1984.
2. Harshbarger, J. C., Charles, A. M., and Spero, P. M. Collection and analysis of neoplasms in sub-homeothermic animals from a phyletic point of view. *In:* C. J. Dawes, J. C. Harshbarger, S. Kondo, T. Sugimura, and S. Takayama (eds.), Phyletic Approaches to Cancer, pp. 357–384. Tokyo: Japanese Science Society Press, 1981.
3. Anders, F., Schartl, M., Barnekow, A., and Anders, A. *Xiphophorus* as an *in vivo* model for studies on normal and defective control of oncogenes. Adv. Cancer Res., *42:* 191–275, 1984.
4. Wittbrodt, J., Adam, D., Malitschek, B., Maueler, W., Raulf, F., Telling, A., Robertson, S. M., and Schartl, M. Novel putative receptor kinase encoded by the melanoma-inducing *Tu* locus in *Xiphophorus*. Nature (Lond.), *341:* 415–421, 1989.
5. Van Beneden, R. J., Watson, D. K., Chen, T-T., Lautenberger, J. A., and Papas, T. S. Cellular *myc* (c-*myc*) in fish (rainbow trout): its relationship to other vertebrate *myc* genes and to the transforming genes of the MC29 family of viruses. Proc. Natl. Acad. Sci. USA, *83:* 3698–3702, 1986.
6. Nemoto, N., Kodama, K., Tazawa, A., Prince Masahito, and Ishikawa, T. Extensive sequence homology of the goldfish *ras* gene to mammalian genes. Differentiation, *32:* 17–23, 1986.
7. Schartl, M., Wittbrodt, J., Maueler, W., Raulf, F., Adam, D., Hannig, G., Telling, A., Storch, F., Andexinger, S., and Robertson, S. M. Oncogenes and melanoma formation in *Xiphophorus* (Teleostei: Poecilliidae). *In:* J. H. Schroder and M. Schartl (eds.), New Trends in Ichthyology. Berlin: Parey Verlag, in press, 1990.
8. Barnekow, A., and Schartl, M. Comparative studies on the *src* proto-oncogene and its gene product pp60$^{c\text{-}src}$ in normal and neoplastic tissues of lower vertebrates. Comp. Biochem. Physiol., *87B:* 663–670, 1989.
9. McMahon, G., Huber, L. J., Stegeman, J. J., and Wogan, G. N. Identification of a c-Ki-*ras* oncogene in a neoplasm isolated from winter flounder. Mar. Environ. Res., *24:* 345–350, 1988.
10. Barbacid, M. *ras* genes. Annu. Rev. Biochem., *56:* 779–827, 1987.
11. Wirgin, I., Currie, D., and Garte, S. J. Activation of the K-*ras* oncogene in liver tumors of Hudson River tomcod. Carcinogenesis (Lond.), *10:* 2311–2315, 1989.
12. Van Beneden, R. J., Henderson, K. W., Gardner, H. S., van der Schalie, W., Blair, D. G., and Papas, T. S. New models for oncogene isolation in the study of carcinogenesis. *In:* Proceedings of the Non-Mammalian Toxicity Assessment Research Review, US Army Biomedical Research and Development Laboratory, in press, 1990.
13. Van Beneden, R. J., Henderson, K. W., Roberts, M. A., Gardner, H. S., and Papas, T. S. Role of oncogenes in chemical carcinogenesis. *In:* Proceedings of the Non-Mammalian Toxicity Assessment Research Review, US Army Biomedical Research and Development Laboratory, in press, 1990.
14. Graham, F. L., and van der Eb, A. J. A new technique for the assay of infectivity of human adeno virus 5 DNA. Virology, *52:* 456–467, 1973.
15. Blair, D. G., Cooper, C. S., Oskarsson, M. K., Eader, L. A., and Vande Woude, G. F. New method for detecting cellular transforming genes. Science (Wash. DC), *218:* 1122–1125, 1982.
16. McPherson, I., and Montegnier, L. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology, *23:* 291–294, 1964.
17. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., *98:* 503–517, 1975.
18. Feinberg, A. P., and Vogelstein, B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., *132:* 6–13, 1983.
19. Schartl, M., Schmidt, C. R., Anders, A., and Barnekow, A. Elevated expression of the cellular *src* gene in tumors of different etiology in *Xiphophotus*. Int. J. Cancer, *36:* 199–207, 1985.
20. Nemoto, N., Kodama, K., Tazawa, A., Matsumoto, J., Masahito, P., and Ishikawa, T. Nucleotide sequence comparison of the predicted first exonic region of goldfish *ras* gene between normal and neoplastic tissues. J. Cancer Res. Clin. Oncol., *113:* 56–60, 1987.
21. Van Beneden, R. J., Watson, D. K., Chen, T. T., Lautenberger, J. A., and Papas, T. S. Teleost oncogenes: evolutionary comparison to other vertebrate oncogenes and possible roles in teleost neoplasms. Mar. Environ. Res., *24:* 339–345, 1988.
22. Dey, W., Peck, T., Smith, C., Cormier, S., and Kraemer, G. L. A study of the occurrence of liver cancer in Atlantic tomcod (*Microgadus tomcod*). Final report to the Hudson River Foundation, NY, 1986.